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(54) Title: CARBOHYDRATE VACCINES FOR VIRAL DISEASES

(57) Abstract

This invention provides a method for treating or preventing a viral disease in a subject comprising administering to the subject an effective amount of a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease. This invention also provides a vaccine for treating or preventing a viral disease comprising a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease, and a pharmaceutically acceptable carrier.

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CARBOHYDRATE VACCINES FOR VIRAL DISEASES

This application claims the benefit of U.S. Provisional Application No. 60/102,657, filed October 1, 1998, the contents of which are hereby incorporated by reference.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Background of the Invention

Dramatic changes in carbohydrate metabolism and cell surface expression have been observed in association with infectious diseases, such as viral infections. For example, in vivo and in vitro infection of human lymphocytes with retroviruses such as the human immunodeficiency virus (HIV) or human T-lymphotropic virus type-1 (HTLV-1) leads to increased cell surface expression of the Lewis y antigen (Adachi et al., J. Exp. Med, 167:323, 1988) and of the gangliosides GM2, GD2, GM1, GM1a, GD1a, and GM3 (Sorice, et al., JAIDS, 12:112, 1996; Misasi et al., Clin. Immunol. Immunopathol., 67:216, 1993; Furukawa et al., PNAS, 90:1972, 1993; Matsuda et al., Biochem. Biophys. Acta, 1168:123, 1993; Auci et al., J. Leukoc. Biol. 52:282, 1992). Increased expression of GM2, GD2 and GM3 was observed in cell lines transfected with human adenovirus genes or infected with a hybrid adenovirus (Sanai et al., J. Biochem. [Tokyo] 107:740, 1990, Jambrosic et al., Int. J. Cancer, 44:1117, 1989).

GM2, GD2 and other gangliosides are sialic acid containing glycosphingolipids composed of a complex carbohydrate moiety linked to a hydrophobic ceramide portion. Embedded within the outer leaflet of the cell membrane, the carbohydrate chain is exposed to the extracellular matrix. The oligosaccharide portion of gangliosides such as GD2 may also be linked to peptide moieties and activate cytotoxic T lymphocytes when presented on the cell surface in

association with major histocompatibility molecules (Zhao and Cheung, J. Exp. Med. 182:67, 1995). The Lewis y antigen is a neutral oligosaccharide found on glycolipids and glycoproteins. A growing number of glycolipids are known to elicit cytotoxic T lymphocyte responses when presented on the cell surface in association with members of the CD1 family of antigen presenting molecules.

As with virally infected cells, a variety of human tumors are characterized by altered expression of carbohydrate structures, including GM2, GD2, and the Lewis y antigen(reviewed in Ragupathi, Cancer Immunol. Immunother. 43:152, 1996). Melanoma patients with naturally occurring antibodies to GM2 have prolonged disease-free and overall survival periods. Cell surface carbohydrate antigens have thus been identified as targets for active and passive immunotherapy of cancers, and different approaches have been adopted to induce immune responses against these structures. These include whole or lysed tumor cells, purified carbohydrates, mimotopes and anti-idiotype antibodies. The immunogenicity of purified carbohydrates can be improved via their conjugation to immunogenic carrier proteins.

While each vaccine approach has shown promise in initial experimentation, adsorption or covalent attachment of purified carbohydrate antigens to immunogenic T-dependent protein carriers is the concept that has been pursued most vigorously, resulting in vaccines that have in some instances been shown to be effective in clinical trials.

In studies aimed at inducing a humoral immune response against gangliosides in melanoma patients by active immunization, GM2/Bacillus Calmette-Guérin (BCG) vaccines were shown to be effective (Livingston et al., Proc. Natl. Acad. Sci. USA 84:2911, 1987; Livingston et al., Cancer Res. 49:4045, 1989). In a randomized study with 122 melanoma patients, who were disease-free after surgery, that it was shown that, out of 64 patients treated with BCG alone and 58

patients with GM2/BCG, the majority of patients (86%) receiving the GM2 vaccine produced antibodies. Patients who produced anti-GM2 antibodies had a significantly longer disease free interval and overall survival than antibody negative patients. (Livingston et al., J. Clin. Oncol. 12:1036, 1994).

Improved humoral immune responses were elicited in humans by a vaccine containing GM2 covalently linked to the carrier protein Keyhole Limpet Hemocyanin (KLH) and administered with the adjuvant QS-21. QS-21 is a carbohydrate extracted from the bark of the South American tree Quillaja saponaria Molina. The monosaccharide composition, molecular weight, adjuvant effect and toxicity for a series of these saponins have been described (Kensil, Crit. Rev. Ther. Drug Carrier Syst. 13:1, 1996). Studies have identified the 100 ug dose of QS-21 as the optimal well tolerated dose for induction of antibodies against GM2 and KLH in humans (Livingston et al., Vaccine 12:1275, 1994).

In the presence of QS-21 adjuvant, the GM2-KLH conjugate vaccine consistently induced high-titer, long-lived IgM responses against GM2 in melanoma patients (Livingston et al., Cancer Immunol. Immunother. 43:324, 1997). In the majority of treated patients, the vaccine also induced anti-GM2 IgG antibodies, which have the ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). The elicited antibodies were shown to specifically bind and kill cancer cells via complement mediated lysis and ADCC.

In addition to gangliosides, other carbohydrate antigens have been made immunogenic by conjugation to appropriate carrier proteins. Coupling of capsular polysaccharides from the bacterium Haemophilus influenzae type b to diphtheria toxiod resulted in a significant increase in immune response and protection (Eskola et al., New England J. Med. 323:1381, 1990). This product has been licensed by the U.S. Food and Drug Administration for use in children and in adults with

splenic dysfunction. Similarly, robust humoral immune responses were elicited in children by pneumococcal polysaccharides conjugated to either tetanus toxoid or the outer membrane protein complex of Neisseria meningitidis (Kayhty et al., J. Infect. Dis. 172:1273, 1995; Anttila et al., J. Infect. Dis., 177:1614, 1998). In addition, vaccination of ovarian cancer patients with synthetic Thompson Friedenreich tumor antigen conjugated to keyhole limpet hemocyanin elicited humoral IgM and IgG responses (MacLean et al., J. Immunotherapy 11:292, 1992). The important finding common in these studies was the isotype switch from a IgM response of short duration to a long lasting, high affinity IgG response indicating that activation of T-cell dependent pathways against carbohydrates is likely to occur.

This approach is now applied to the use of ganglioside and other carbohydrate-based vaccines for the treatment or prevention of viral infections wherein carbohydrate metabolism and expression are altered. The goal is to similarly induce immune responses to carbohydrate antigens associated with diseased cells. Through well known mechanisms, the immune system can destroy the infected cells or viruses that display the carbohydrate antigens and thereby prevent or favorably alter the course of the viral infection.

Antibodies are known to be an important component of the host defense against viruses (Klein, Immunology, Blackwell Scientific Publications, Boston, MA, 1990). Antibodies can bind to viruses and eliminate their infectivity by direct neutralization, complement-mediated virolysis, or Fc receptor-mediated phagocytosis. In addition, antibodies can eliminate virally infected cells via either complement-mediated cytolysis or antibody-dependent cell-mediated cytotoxicity. Incorporation of host cell antigens into virus particles is a well-established phenomenon, and it has been further demonstrated that protection from viral

infections can be afforded by the immunological response to host cell antigens as well as virally encoded antigens (Schultz and Stott, AIDS, 8:[suppl 1]:5203, 1994).

Similarly, cell-mediated immunity is known to play a major role in controlling viral infections. Importantly, cytotoxic T lymphocytes can eliminate virally infected cells wherein antigen expression is altered. As described above, cytotoxic T lymphocytes can recognize carbohydrate antigens that are either lipid-linked and presented in association with CD1 molecules or peptide-linked and presented in association with major histocompatibility molecules.

Summary of the Invention

This invention provides a method for treating or preventing a viral disease in a subject comprising administering to the subject a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease. In an embodiment, the viral disease is caused by the human immunodeficiency virus type-1.

In an embodiment, the carbohydrate is the Lewis y antigen, a ganglioside, or the oligosaccharide portion of a ganglioside. The gangliosides may include, but not to be limited to, GM1, GM1a, GM2, GM3, GD1a, and GD2. In a preferred embodiment, the ganglioside is GM2, GD2 or a combination thereof.

In an embodiment, the molecular mimic is an anti-idiotype antibody. In another embodiment, the molecular mimic is a peptide mimotope.

In an embodiment, the cellular carbohydrate antigen or a molecular mimic thereof is administered to the subject together with a suitable adjuvant. In a preferred embodiment, the adjuvant is QS-21.

In a further embodiment, the cellular carbohydrate antigen or a molecular mimic thereof is adsorbed, covalently linked or otherwise conjugated to an immunogenic carrier protein. In a preferred embodiment, the carrier protein is Keyhole Limpet Hemocyanin.

This invention also provides a vaccine for treating or preventing a viral disease in a subject comprising administering to the subject a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral

disease, and a pharmaceutically acceptable carrier.

Detailed Description of the Invention

This invention provides a method for treating or preventing a viral disease in a subject comprising administering to the subject a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease.

As used herein, the molecular mimic is defined as a compound which when administered to an appropriate host, stimulates or enhances an immune response that recognizes the cellular carbohydrate. Such mimics include, but are not limited to, mimotopes and anti-idiotype antibodies. Mimotopes are peptides which mimic the immunogenicity of the cellular carbohydrate. It is known that anti-idiotype antibodies can mimic carbohydrate antigens for the purposes of inducing anti- carbohydrate immune responses. For example, U.S. Patent No. 5,792,455, entitled, "Anti-idiotype antibody vaccine," describes an anti-idiotype antibody which mimics the ganglioside GD3; and U.S. Patent No. 5,653,977, entitled, "Anti-idiotypic antibody that mimics the GD2 antigen," describes an anti-idiotypic monoclonal antibody which elicits an immune response reactive against the ganglioside GD2 antigen. The content of these patents is incorporated into this application by reference.

In an embodiment, the cellular carbohydrate antigen or a molecular mimic thereof is administered to the subject in the presence of a suitable adjuvant. Suitable adjuvants include the precipitated aluminum salts collectively known as alum, cytokines such as IL-2 and interferon-gamma, block copolymer-based adjuvants such as titermax, Ribi Detox and other monophosphoryl lipid A containing adjuvants, and saponins such as QS-21. In a preferred embodiment, the adjuvant is QS-21.

In an embodiment, the viral disease is caused by human immunodeficiency virus type-1

In an embodiment, the cellular carbohydrate antigen is the Lewis y antigen, a ganglioside, or the oligosaccharide portion of a ganglioside. The gangliosides which are applicable to this invention include, but are not limited to, GM1, GM1a, GM2, GM3, GD1a, and GD2. In a preferred embodiment, the ganglioside is GM2, GD2 or a combination thereof.

In an embodiment, the cellular carbohydrate antigen or a molecular mimic thereof is adsorbed, covalently linked or otherwise conjugated to an immunogenic carrier protein.

Ganglioside conjugate vaccines have been described. See e.g. Livingston and Helling, "Ganglioside-KLH Conjugate Vaccines with QS-21" Patent Cooperation Treaty (PCT) Application No: PCT/US94/00757, International Publication Number: WO/94/16731, the content of which is incorporated into this application by reference.

In a preferred embodiment of this invention, the conjugated ganglioside is GM2, GD2 or a combination thereof.

Different effective amounts of the conjugated ganglioside or oligosaccharide portion thereof may be used according to this invention. A person of ordinary skill in the art can perform simple titration experiments to determine what amount is required for effective immunization. An example of such titration experiment is to inject different amounts of the conjugated ganglioside or conjugated oligosaccharide portion thereof to the subject with or without a suitable adjuvant, and then examine the immune response.

In an embodiment, the effective amount of conjugated ganglioside or conjugated oligosaccharide portion thereof is an amount between about 1 μg and about 500 μg.

In another embodiment, the effective amount of conjugated ganglioside or conjugated oligosaccharide portion thereof is

an amount between about 50 μg and about 90 μg. In an embodiment, the effective amount of conjugated ganglioside or conjugated oligosaccharide portion thereof is about 70 μg.

In another embodiment, the effective amount of conjugated ganglioside or conjugated oligosaccharide portion thereof is between about 1 μg and about 10 μg. In a more specific embodiment, the effective amount of conjugated ganglioside or conjugated oligosaccharide portion thereof is between about 7 μg and about 10 μg. In an embodiment, the effective amount of conjugated ganglioside or conjugated oligosaccharide portion thereof is about 7 μg.

In addition, the effective amount of the adjuvant may also be similarly determined, i.e. by administering different amounts of the adjuvant with the cellular carbohydrate antigen or a molecular mimic thereof and examining the immune response so as to determine which amount is effective. When using QS-21 as adjuvant, the effective amount of QS-21 may also to be similarly determined.

In a preferred embodiment, the effective amount of QS-21 is an amount between about 10μg and about 200 μg. In an embodiment, the effective amount of QS-21 is about 100 μg. In another embodiment, the effective amount of QS-21 is about 200 μg.

In a preferred embodiment, the cellular carbohydrate antigen or a molecular mimic thereof is conjugated to an immunogenic protein. As used herein, an immunogenic protein is a polypeptide that, when conjugated to a carbohydrate or a molecular mimic thereof stimulates or enhances an immune response to the carbohydrate in the subject. In a further embodiment, the immunogenic protein is Keyhole Limpet Hemocyanin or a derivative thereof. This invention also provides the above-described vaccine wherein the cellular carbohydrate antigen or a molecular mimic thereof is

conjugated to Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin.

Keyhole Limpet Hemocyanin is a well-known protein. A derivative of Keyhole Limpet Hemocyanin may be generated by direct linkage of at least one immunological adjuvant such as monophospholipid A or non-ionic block copolymers or cytokines to Keyhole Limpet Hemocyanin. Cytokines are well known to an ordinary skilled practitioner. Example cytokines with adjuvant properties include interleukin 2 and interferon-gamma. There are other known cytokines in the art which may to be linked to Keyhole Limpet Hemocyanin, forming a derivative of Keyhole Limpet Hemocyanin.

In an embodiment, a ganglioside is conjugated to the immunogenic protein by the process of reductive amination following oxidation of a ceramide alkene structure to an aldehyde. In another embodiment, the conjugation of the ganglioside occurs through an aminolysyl group of the Keyhole Limpet Hemocyanin.

In addition to reductive amination, various other conjugation techniques may to be used for this invention. The conjugation techniques may include chemical linker groups and should not adversely affect the immunogenicity of the carbohydrate.

As used herein, a suitable adjuvant is an adjuvant which when administered together with the carbohydrate or a molecular mimic thereof or a conjugated carbohydrate or a conjugated molecular mimic thereof stimulates or enhances an immune response to the carbohydrate in the subject. In an embodiment, the adjuvant is QS-21. There are other known adjuvants which may to be applicable to this invention. There may be classes of QS-21 or QS-21 like chemicals which may be similarly used in accordance with this invention.

This invention also provides a vaccine for treating or

preventing a viral disease comprising a cellular carbohydrate antigen that is overexpressed during the viral infection, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease, and a pharmaceutically acceptable carrier.

In an embodiment of this invention, the subject is a human.

In an embodiment, the cellular carbohydrate antigen is the Lewis y antigen, a ganglioside or the oligosaccharide portion of a ganglioside.

This invention further provides a vaccine for stimulating or enhancing in a subject to which the vaccine is administered, an immune response which recognizes a cellular carbohydrate antigen that is overexpressed during a viral disease comprising an amount of such carbohydrate or a molecular mimic thereof effective to stimulate or enhance an anti-carbohydrate immune response in the subject, and a pharmaceutically acceptable vehicle, wherein the subject is afflicted with the viral disease and the immune response produced in the subject upon administration of the vaccine effectively treats the viral disease.

This invention also provides a vaccine for stimulating or enhancing in a subject to which the vaccine is administered, an immune response which recognizes a cellular carbohydrate antigen that is overexpressed during a viral disease comprising an amount of such carbohydrate or molecular mimic thereof effective to stimulate or enhance an anti-carbohydrate immune response in the subject, and a pharmaceutically acceptable vehicle, wherein the subject is susceptible to the viral disease and the immune response produced in the subject upon administration of the vaccine effectively prevents the viral disease.

This invention further provides a vaccine for a viral

disease, wherein the virus or the virus-infected cells have gangliosides on their surface.

This invention further provides a method for treating viral diseases in a subject afflicted with a viral disease comprising administering to the subject an effective dose of a vaccine for stimulating or enhancing in a subject to which the vaccine is administered, production of an antibody which recognizes a ganglioside, comprising an amount of ganglioside or oligosaccharide portion thereof conjugated to an immunogenic protein effective to stimulate or enhance antibody production in the subject, an effective amount of adjuvant and a pharmaceutically acceptable vehicle, wherein the subject is afflicted with a viral disease and the antibody produced in the subject upon administration of the vaccine effectively treats the viral disease.

This invention further provides a method for preventing a viral disease in a subject susceptible to a viral disease comprising administering to the subject an effective dose of a vaccine for stimulating or enhancing in a subject to which the vaccine is administered, production of an antibody which recognizes a ganglioside, comprising an amount of ganglioside or oligosaccharide portion thereof conjugated to an immunogenic protein effective to stimulate or enhance antibody production in the subject, an effective amount of adjuvant and a pharmaceutically acceptable vehicle, wherein the subject is susceptible to a viral disease and the antibody produced in the subject upon administration of the vaccine effectively prevents the viral disease.

This invention also provides a method of using the above-described vaccine, wherein the ganglioside or oligosaccharide portion thereof is conjugated to Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin. This invention further provides a method of using the above-described vaccine wherein the adjuvant is QS-21.

This invention further provides a method of using the above-described vaccine for treating or preventing viral disease, wherein the virus or virus-infected cells have gangliosides on their surface.

For the purposes of this invention "pharmaceutically acceptable vehicles" means any of the standard pharmaceutical vehicles. Examples of suitable vehicles are well known in the art and may include, but not limited to, any of the standard pharmaceutical vehicles such as a phosphate buffered saline solutions, phosphate buffered saline containing Polysorb 80, water, emulsions such as oil/water emulsion, and various types of wetting agents.

The vaccine of this invention may be administered intradermally, subcutaneously and intramuscularly. Other methods well known by a person of ordinary skill in the art may also be used.

In a preferred embodiment this invention provides a method for stimulating or enhancing in a subject production of antibodies which recognize a ganglioside comprising administering to the subject an effective dose of a vaccine for stimulating or enhancing in a subject to which the vaccine is administered, production of an antibody which recognizes a ganglioside, comprising an amount of ganglioside or oligosaccharide portion thereof conjugated to an immunogenic protein effective to stimulate or enhance antibody production in the subject, an effective amount of adjuvant and a pharmaceutically acceptable vehicle, wherein the administering comprises administering the effective dose at two or more sites. "Administering the effective dose at two or more sites" means that the effective dose is divided into two or more portions and each portion is administered at a different site of the subject. In a specific embodiment, the administering comprises administering at three sites.

This invention will to be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Flow cytometric analysis of antibody binding to HIV-1 infected cells

Approximately 10^6 HIV-infected or control cells are stained first with approximately 50μl of anti-carbohydrate antibody (~5 μg/mL) for approximately 20 min at 4 °C in PBS/1%FBS/0.1% sodium azide (assay buffer) washed with assay buffer and then stained with a phycoerythrin-conjugated reporter antibody for 20 minutes at 4 °C. The cells are washed with assay buffer, fixed overnight at 4°C with PBS/1% FBS/1% formaldehyde, and analyzed on a flow cytometer. The GD2- and GM2-positive melanoma cell line SK-MEL-31 can serve as a positive control in assays using anti-GM2 and anti-GD2 antibodies.

The antibodies may to be monoclonal antibodies, purified hyperimmune immunoglobulin, or human or animal sera known to contain high levels of carbohydrate-reactive antibodies produced naturally or elicited by active immunization. The HIV-1 infected cells may be either cell lines competent for HIV-1 infection or phytohemagglutinin-stimulated human peripheral blood mononuclear cells (PBMC). The HIV-1 viruses may include laboratory adapted X4 viruses and primary R5, X4 and R5X4 isolates.

Complement-mediated lysis of HIV-1 infected cells

Complement-mediated cytotoxicity assays are performed by a 4h ^{51}Cr release assay. 2x10^6 cells are labelled with 100μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) in 10% FCS RPMI for 1h at 37°C in a CO_2 incubator. The cells are washed twice, and 10^4 cells/well in 96-well round-bottom plates

(Corning, New York, NY) are labelled and incubated with sera, antibodies or with medium alone for 1h at 37°C in a CO_2 incubator. The cells are washed and incubated with human complement (Sigma) at a dilution of 1:4 for 4h at 37°C. The plates are spun at 500g for 5 min, and a 125µl aliquot of supernatant of each well is harvested for determination of released ^{51}Cr. All assays are performed in triplicate and include control wells for maximum release in 1% NP-40 (Sigma) and for spontaneous release in the absence of complement. The GD2- and GM2-positive melanoma cell line SK-MEL-31 can serve as positive control target cells for assays using anti-GM2 and anti-GD2 antibodies.

The percentage of specific lysis is calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release - spontaneous release}}{\text{Maximum release - spontaneous release}} \times 100$$

The antibodies may to be monoclonal antibodies, purified hyperimmune immunoglobulin, or human or animal sera known to contain high levels of carbohydrate-reactive antibodies produced naturally or elicited by active immunization. The HIV-1 infected cells may be either cell lines competent for HIV-1 infection or PBMC. The HIV-1 viruses may include laboratory adapted X4 viruses and primary R5, X4 and R5X4 isolates.

Anti-carbohydrate antibody-dependent cell-mediated cytotoxicity (ADCC) assay

PBMC are suspended in RPMI media supplemented with 10% heat-inactivated FBS (assay media) at a density of $4x10^6$ cells/ml. These cells are then incubated overnight at 37°C prior to use as effectors in the ADCC assay. The following day, target cells are labeled with $Na_2{}^{51}CrO_4$ (100µCi per $2x10^6$ cells) for approximately 3 h in RPMI 1640 assay media prior to use in the assay. In each well, 10^4 ^{51}Cr-labeled target cells are preincubated with various dilutions of sera or concentrations of antibodies in 150µl total assay volume for

30 min at 37°C. Then 10^6 PBMC are added in 50µl assay media, and the plates are centrifuged at 300g for 5 minutes prior to incubation at 37°C for 4h. Plates are then centrifuged at 300g, and 100µl aliquots of cell supernatants are collected. ^{51}Cr release is detected using a Wallac 1470 gamma counter.

All assays are performed in triplicate and include control wells for maximum release in 1% NP-40 (Sigma) and for spontaneous release in the absence of complement.

The percentage of specific lysis is calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release - spontaneous release}}{\text{Maximum release - spontaneous release}} \times 100$$

The antibodies may to be monoclonal antibodies, or purified hyperimmune immunoglobulin, or human or animal sera known to contain high levels of carbohydrate-reactive antibodies produced naturally or elicited by active immunization. The HIV-1 infected cells may be either cell lines competent for HIV-1 infection or PBMC. The HIV-1 viruses may include laboratory adapted X4 viruses and primary R5, X4 and R5X4 isolates. The GD2- and GM2-positive melanoma cell line SK-MEL-31 can serve as positive control target cells for assays using anti-GM2 and anti-GD2 antibodies.

HIV-1 neutralization assays

Virus neutralization is assessed using phytohemagglutinin-stimulated PBMC as indicator cells, with determination of p24 antigen production as the endpoint. PBMC are stimulated with PHA for 48 h before removal of the mitogen by washing. Antibodies are combined in 2-fold serial dilutions with virus and/or cells for 1 h at 37 °C. The virus is then added to the PBMC at a density of 4 x 10^6 /ml and the cultures incubated for 7 days. The culture supernatants are harvested, treated with 1% Empigen detergent before determination of the p24 concentration by ELISA. The Leu

3a HIV-1 inhibitory monoclonal antibody (Becton Dickinson) may to be used as a positive control.

The antibodies may to be monoclonal antibodies, purified hyperimmune immunoglobulin, or human or animal sera known to contain high levels of carbohydrate-reactive antibodies produced naturally or elicited by active immunization. The HIV-1 viruses may include laboratory adapted X4 viruses and primary R5, X4 and R5X4 isolates.

Preparation of Clinical Grade Ganglioside Conjugate Vaccine

CHEMISTRY AND MANUFACTURING

DRUG SUBSTANCE

NAME AND SOURCE

Proper name:

GM2-KLH

Chemical name:

11^{3}NeuAc-GgOse$_3$Cer-keyhole limpet hemocyanin (KLH)

Manufacturer: Progenics Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591, United States of America.

MATERIALS USED FOR THE PREPARATION OF GM2

MATERIAL	SUPPLIER	GRADE
Acetone	BDH	ACS
Ammonia Solution	BDH	ACS
Chloroform	BDH	ACS
Ethanol	Commercial Alcohol Ltd.	---
Ethyl Ether	BDH	ACS
Methanol	BDH	ACS
2-Propanol	Fisher UN1219	ACS
Water	Travanol sterile water for irrigation.	
Calcium Chloride (anhydrous - 20 mesh granular)	Fisher	Certified

Dimethyl Sulfide	Aldrich	99% +
GM2	Fidia	---
Oxygen	Linde UN1072	USP
Silica Gel	E Merck	Kieselgel 60H Art 7736
Sodium Cyanoborohydride	Aldrich	95% Pure
TLC Plates	E Merck	Kieselgel 60H F254

MATERIALS USED IN THE CONJUGATION PROCEDURE

MATERIAL	SUPPLIER	GRADE
Keyhole limpet hemocyanin (KLH)	Perimmune, Inc. Rockville, MD	USP
Deoxycholic acid, sodium salt (DOC) (monohydrate) 98%	Aldrich	Analytical
Ethylenediamine tetraacetic acid	Aldrich	ACS
di-sodium hydrogen orthophosphate (anhydrous) (Na_2HPO_4)	BDH	Analytical
Sodium chloride (NaCl)	BDH	Analytical
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH	Analytical
Sodium hydroxide (NaOH)	BDH	Analytical
Tris (hydroxymethyl) aminomethane hydrochloride	Sigman	_____
Sodium cyanoborohydride ($NaBH_3CN$)	Aldrich	_____
Sepharose CL-4B	Pharmacia	_____
Nitrogen gas (filtered)	Medigas	_____
GM2 aldehyde	Progenics	_____

DEVELOPMENT CHEMISTRY

Data for the GM2 and GM2 Aldehyde:

The structures of GM2 and GM2 aldehyde were characterized by ^1H NMR spectroscopy, thin layer chromatography (TLC), FAB-MS

and FT-IR.

STRUCTURAL FORMULA	MOLECULAR FORMULA	MOLECULAR WEIGHT
GM2 - ganglioside (compound #1)		
GalNAcB1-4GaLB1-4 GlcB1-1Cer Neu5Aca2	$C_{67}H_{121}O_{26}N_3$	M-1=1382 Solid
I^3NeuAc-GgOse$_3$Cer	$C_{69}H_{125}O_{26}N_3$ (acid)	M-1=1410

TLC: Rf=0.21 (65:35:8 $CHCL_3$-CH_3OH-H_2O)
Rf=0.60 (5:4:1 $CHCl_3$-CH_3OH-0.2% aqueous $CaCl_2$)
Rf=0.2 (7:1:1 $(CH_3)_2CHOH$-NH_4OH-H_2O)

STRUCTURAL FORMULA	MOLECULAR FORMULA	MOL. WT.	PHYSICO-CHEMICAL CHARACTERISTICS
GM2-aldehyde (compound #2)	$C_{53}H_{93}O_{27}N_3$	1204.29	Cream White, Odorless, Amphorous Solid

STRUCTURAL DATA

^{1}H(DMSO-d_6:D_2) δ:9.48(d,1H,J=2,OHz), 4.79(d,1H,J=8.5Hz, III-1), 4.26(d,1H,J=8.OHz,II-1), 4.19 (d,1H,J=8,OHZ,I-1),2.54(dd,1H, A-3e), 1.88(s,3H,Ac), 1.78(s,3H,Ac),0.85(t,3H,J=6.6Hz,CH_3).

FT-IR (KBr Cast, CM^{-1}): 3439, 3420, 2952, 2923, 2851, 1634, 1070 (possibly the gem diol).

TLC Rf=0.5 (5:4:1 $CHCl_3$-CH_3OH-0.2% aquecous $CaCl_2$)

Data for KLH and GM2-KLH:

Keyhole limpet hemocyanin (KLH) is a large, complex protein composed of a number of smaller molecular weight subunits. KLH is extracted and purified from the keyhole limpet mollusk (Megathura crenulata).

COMPOUND	SEPHAROSE CL-4B CHROMATOGRAPHY Molecular Weight (daltons)	ISOELECTRIC FOCUSING (Isoelec. pts.)	RESORCINOL-GEL HCl moles of GM2/ moles of protein
KLH	Whole mol.(2):>2x10^6 Subunits: 2-7 x 10^5	Mult. bands between pH 4.65	

	and pH 6		
GM2-KLH	Whole mol.(2)>2x10^6 Subunits: 2-7 x10^5	Multiple bands between pH 4.65 and pH 6	200- 1400

GM2-KLH MANUFACTURING FLOW CHART

STEP 1 - PURIFICATION OF GM2:

GM2 (FIDIA)

↓

Silica Gel Column Chromatography

1. 65:35 chloroform - methanol
2. 65:35:4 chloroform - methanol - water

↓

In-process QC:

1. TLC

↓

STEP 2 - SYNTHESIS OF GM2 ALDEHYDE (COMPOUND #2):

GM2 (Compound #1)

↓

(1) O_3, MeOH

↓

(2) CH_3SCH_3

↓

GM2 Aldehyde (Compound #2, may be the gem diol)

↓

In-process tests done in-house:

1. TLC

↓

STEP 3 - CONJUGATION OF THE GM2 ADEHYDE TO KLH:

Sterile pyrogen free KLH

↓

KLH added to GM2 aldehyde in 4:1 ratio (w/w)

↓

Incubated at room temperature with shaking for 3 minutes

↓

$NaBH_3CN$ is added to GM2 Adehyde/KLH mixture in 1:1 ratio (w/w)

↓

Reaction mixture is gently stirred at room temperature

overnight then at 40°C for 4 days

↓

STEP 4 - DIAFILTRATION OF THE CONJUGATE:

Conjugate is diafiltered vs.:

-PBS pH 7.5
-TRIS/EDTA pH 7.75
-TRIS/EDTA/0.05% DOC pH 7.75
-TRIS/EDTA pH 7.5
-PBS pH 7.5

Conjugate aseptically removed from the Amicon filtration unit

↓

Centrifuged

↓

Conjugate sterile filtered

↓

In-process QC tests:

1. BioRad Protein Assay
2. Sepharose gel filtration
3. Isoelectric focusing (IEF)

↓

Concentration of conjugate aseptically adjusted to 1 mg/mL

↓

Conjugate dispensed into 1 mL sterile, pyrogen free vials and stored at 2-8°C

↓

Final QC testing:

1. Enzyme immunoassay (EIA)
2. LAL pyrogen test
3. BioRad protein assay
4. Resorcinol-HCl assay
5. Rabbit pyrogen test
6. General safety test
7. Sterility test
8. Impurity test for cyanide

METHOD OF MANUFACTURE OF GM2-KLH CONJUGATE

The manufacturing of the GM2-KLH conjugate is carried out in 4 steps:

1. Purification of incoming GM2 (bovine source) (compound #1).

2. Synthesis of GM2 aldehyde (compound #2).
3. Conjugation of the GM2 aldehyde to KLH.
4. Diafiltration of the conjugate.

Step 1: Purification to GM2 (Compound #1):

Name: GM2 ganglioside

Abbreviated Name: $II^3NeuAc\text{-}GgOse_3Cer$

GM2 ganglioside (bovine source) starting material is supplied by FIDIA. All glass ware is washed with distilled acetone followed by distilled ethanol and then dried (130°C) for 18 hours prior to use. A column (Michel-Miller S 795-10) of silica gel (30.5g, Kieselgel 60H, Art 7736, E. Merck) is packed at 75 psi (SSI Model 300 Lo pump) using 65:35 chloroform:methanol as solvent. GM2 (200 mg) is applied as a concentrated 65:35 chloroform-methanol solution and elution is performed with this solvent, followed by 65:35:4 chloroform-methanol-water. The fractions are analyzed by TLC (Rf 0.6, 5:4:1 chloroform-methanol-0.2% aqueous $CaCl_2$). The GM2 containing fractions are pooled and evaporated to give a creamy white amorphous solid.

In-process testing for this material (compound #1) includes ^1H NMR and thin layer chromatography (TLC) to confirm the identity and purity of this ganglioside. The in-process test results must meet the specifications listed under developmental chemistry. If this material is found to be impure, the above purification is repeated.

Step 2: Synthesis of GM2 Aldehyde (Compound #2):

All glassware is rinsed with distilled methanol and dried (130°C) for 18 hours prior to use. A solution of the purified GM2 ganglioside (compound #1) (40 mg) in distilled methanol (10 mL) is stirred at −15°C (dry ice-ethanol) and ozone gas (Orec O3V10-0 ozonator) is passed through the solution for 7 minutes. A stream of argon is then passed through the solution while the reaction is checked by TLC

(5:4:1 chloroform-methanol- 0.2% aqueous $CaCl_2$). The solvents are then removed under reduced pressure and the resulting material is dissolved in distilled methanol. To this solution is added methylsulfide (200 ml) and the reaction mixture is stirred at room temperature for one hour. The solvents are then removed and the residue is washed with ethyl ether(4 x 25 mL). The resulting white solid (compound #2) is dried in vacuo for 15 minutes to remove any remaining solvent and is then used directly in the subsequent conjugation step.

Due to the unstable nature of the resulting aldehyde (B-elimination), compound #2 is identified on a routine basis only by TLC. The TLC of a typical run generally indicates the presence of a small amount of sphinganine or phytosphingosine analog (same Rf as compound #1) and a small amount of reducing sugar (Rf 0.32).

Step 3: Conjugation of GM2 Aldehyde to KLH:

All manipulations are done in a Class 100 biological safety cabinet.

The KLH protein (160 mg) is aseptically measured and added to the flask containing the lyophilized GM2 Aldehyde and a magnetic stir bar. The solution is gently agitated at room temperature for 3 minutes until all of the GM2 Aldehyde has gone into solution.

The sodium cyanoborohydride ($NaBH_3CN$) (40 mg) is added to the GM2 Aldehyde/KLH solution then the flask is sealed with a stopper equipped with a sterile filter needle. The solution is gently shaken then incubated overnight at room temperature. The solution is then further incubated at 40°C for 4 days.

Step 4: Diafiltration of the Glycoconjugates (GM2-KLH):

The contents of the GM2/KLH reaction vial are aseptically transferred to a sterile, pyrogen-free Amicon ultrafiltration unit with a YM-30 filter. Filtered nitrogen is used to provide an operating pressure of 16 psi for the Amicon unit. The conjugate is then diafiltered against the following sterile, pyrogen-free or low pyrogen content buffers successively:

1. 2 complete changes of PBS pH 7.5 (sterile, pyrogen-free)
2. 2 complete changes of TRIS-HCI, EDTA pH 7.75 (sterile, low pyrogen content)
3. 2 complete changes of TRIS-HCI pH 7.75 with 0.5% Deoxycholic acid (DOC) (sterile, low pyrogen content)
4. 4 complete changes of TRIS-HCI pH 7.75 (sterile, low pyrogen content)
5. 3 complete changes of PBS pH 7.5 (sterile, pyrogen-free)

The glycoconjugate is then aseptically removed from the filtration unit and spun at 2000 rpm for 30 minutes. The supernatant is then sterile filtered with a 0.22 mm low protein binding filter.

A sample of the glycoconjugate is obtained and the following in-process QC tests are done:

1. Sepharose gel filtration
2. Isoelectric focusing (IEF)
3. BioRad protein assay

Based on the results of the protein assay, the final volume of the glycoconjugate is adjusted with sterile, pyrogen-free pH 7.5 PBS buffer to yield a protein concentration of 1mg/mL.

Inside of a Class 100 biological safety cabinet, the final glycoconjugate is then dispensed in 1.0 mL aliquots with an overfill volume of 0.1 mL into 2 mL sterile, pyrogen-free, clear, borosilicate serum vials with rubber stoppers and

stored at 2-8°C. During the filling procedure, the air inside the filing area is monitored by exposing two blood agar plates to the air near the work area inside of the hood for a minimum of thirty minutes. These plates are then transferred to a 37°C incubator and incubated for 1-2 days. The plates are then examined for any bacterial or fungal colonies.

The product is labeled by the manufacturing personnel and the labeling is verified by the Quality Control department. The product is then stored at 2-8°C.

Each lot of GM2 - KLH goes through the following Final Quality Control tests:

1. Enzyme Immunoassay (EIA)
2. LAL pyrogen test
3. BioRad protein assay
4. Resorcinol-HCl carbohydrate assay
5. Rabbit pyrogen test
6. General safety test
7. Sterility test
8. Impurity testing for cyanide

What is claimed is:

1. A method for treating or preventing a viral disease in a subject comprising administering to the subject a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease.

2. The method of claim 1, wherein the subject is a human.

3. The method of claim 1, wherein the viral disease is caused by the human immunodeficiency virus type-1.

4. The method of claim 1, wherein the carbohydrate is the Lewis y antigen, a ganglioside or the oligosaccharide portion of a ganglioside.

5. The method of claim 4, wherein the ganglioside is GM1, GM1a, GM2, GM3, GD1a, or GD2 or a combination thereof.

6. The method of claim 4, wherein the ganglioside is GM2 or GD2 or a combination thereof.

7. The method of claim 1, wherein the cellular carbohydrate antigen or a molecular mimic thereof is conjugated to a carrier protein.

8. The method of claim 7, wherein the carrier protein is Keyhole Limpet Hemocyanin or a derivative thereof.

9. The method of claim 1, wherein the cellular carbohydrate antigen or a molecular mimic thereof is administered to the subject together with a suitable adjuvant.

10. The method of claim 9, wherein the adjuvant is QS-21.

11. The method of claim 6, wherein the viral disease is caused by the human immunodeficiency virus type-1.

12. The method of claim 11, wherein the ganglioside or oligosaccharide portion thereof is conjugated to Keyhole Limpet Hemocyanin or a derivative thereof.

13. The method of claim 12, wherein the conjugated ganglioside or oligosaccharide portion thereof is administered together with the adjuvant QS-21.

14. The method of claim 1, wherein the molecular mimic is an anti-idiotype antibody.

15. The method of claim 1, wherein the molecular mimic is a peptide mimotope.

16. A vaccine for treating or preventing a viral disease comprising a cellular carbohydrate antigen that is overexpressed during the viral disease, or a molecular mimic thereof, the amount of such carbohydrate or such mimic being effective to treat or prevent the viral disease, and a pharmaceutically acceptable carrier.

17. The vaccine of claim 16, wherein the carbohydrate is the Lewis y antigen, a ganglioside or the oligosaccharide portion of a ganglioside.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23013

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 39/21
US CL :424/184.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

AIDSLINE, MEDLINE, USPATFUL, EUROPATFUL, WEST-WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 87/06840 A1 (SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH) 19 November 1987, see entire document.	1-17
A	US 5,102,663 A (LIVINGSTON et al.) 07 April 1992, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 JANUARY 2000	10 FEB 2000
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